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Request for grant of a patent

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1. Your reference — SMC 60566/GB/P1

2. Patent application number *(The Patent Office will fill in this part)* — 0229423.9 — 18 DEC 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — Avecia Limited
Hexagon House
Blackley
Manchester, M9 8ZS

Patents ADP number *(if you know it)* — 07764137001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — Process

5. Name of your agent *(if you have one)* — REVELL, Christopher

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* —
Avecia Limited
Hexagon House
PO Box 42
Blackley
Manchester M9 8ZS

Patents ADP number *(if you know it)* — [redacted]01 6997084002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
   a) *any applicant named in part 3 is not an inventor, or*
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Continuation sheets of this form	
Description	4
Claim(s)	
Abstract	
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10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 19/12/02

Avecia Limited Authorised Signatory

12. Name and daytime telephone number of person to contact in the United Kingdom

K.M.Pinder/G.Terry 0161 721 1361/2

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PROCESS

The present invention concerns a process for the purification of nucleoside phosphoramidites.

Synthetic oligonucleotides are important diagnostic tools for the detection of genetic and viral diseases. In addition, oligonucleotides and modified oligonucleotides are of interest as therapeutic candidates that inhibit gene expression or protein function. Large scale synthesis of oligonucleotides for use as therapeutic candidates has become increasingly important since FDA approval of an oligonucleotide analog for the treatment of cytomegalovirus (CMV), and several other oligonucleotide analogs are currently in clinical trials. Kilogram quantities of a purified oligonucleotide analog are needed for each clinical trial.

The principal method currently employed for the preparation of oligonucleotide is the phosphoramidite approach. The increasing demand for larger quantities of oligonucleotides has correspondingly increased demand for phosphoramidite compounds. Phosphoramidite compounds are commonly prepared by phosphitylation of a nucleoside with a phosphitylation agent in the presence of an activator. Hitherto, phosphoramidites have been purified by the use of lengthy and time consuming chromatography.

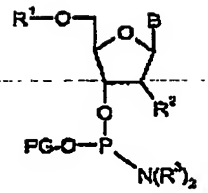
Alternative methods to purify phosphoramidites, especially methods applicable to large scale phosphoramidite preparation are therefore necessary.

According to a first aspect of the present invention, there is provided a process for the purification of an oligonucleotide synthon, which comprises subjecting a solution comprising an oligonucleotide synthon and lower molecular weight impurities to nanofiltration whereby the ratio of an oligonucleotide synthon to lower molecular weight impurities in the solution is increased after the nanofiltration.

Oligonucleotide synthons which can be purified by the process according to the present invention include nucleoside or oligonucleotide phosphoramidites, nucleoside or oligonucleotide H-phosphonates, especially 3'- or 5'-terminal ribo or deoxyribonucleoside H-phosphonate monoesters, and nucleoside or oligonucleotide phosphoramidates.

The process according to the present invention is advantageously employed to purify protected nucleoside phosphoramidites. Preferred protected nucleoside phosphoramidites are deoxyribonucleside-3'-phosphoramidite or ribonucleside-3'-phosphoramidites. The invention is equally applicable to 5'-phosphoramidites.

Examples of preferred protected nucleoside phosphoramidites are compounds of formula (1):



wherein R^1 is a protecting group, preferably a trityl, monomethoxytrityl or dimethoxytrityl group, B is a nucleoside base, R^2 represents -H, -F $-OR^4$, $-NR^5R^6$, $-SR^7$, or a substituted or unsubstituted aliphatic group, such as methyl or allyl. PG is a phosphorus protecting group, commonly a cleavable phosphorus protecting group employed in oligonucleotide synthesis, and preferably a substituted or unsubstituted aliphatic group or a group of formula $-OCH_2CH_2CN$, $-SCH_2CH_2CN$, $-OR^8$, $-SR^8$, $-O-CH_2CH_2-Si(CH_3)_2C_6H_5$, $-O-CH_2CH_2-S(O)_2-CH_2CH_3$, $-O-CH_2CH_2-C_6H_4-NO_2$, $-S-CH_2CH_2-Si(CH_3)_2C_6H_5$, $-S-CH_2CH_2-S(O)_2-CH_2CH_3$; or $-S-CH_2CH_2-C_6H_4-NO_2$. R^4 represents -H, a substituted or unsubstituted aliphatic group (e.g., methyl, ethyl, methoxyethyl or allyl), a substituted or unsubstituted aryl group, a substituted or unsubstituted aralkyl, an alcohol protecting group, especially a base-labile or a silyl protecting group, or $-(CH_2)_q-NR^9R^{10}$. R^5 and R^6 are each, independently, -H, a substituted or unsubstituted aliphatic group, or an amine protecting group. Alternatively, R^5 and R^6 taken together with the nitrogen to which they are attached are a heterocyclyl group. R^7 represents -H, a substituted or unsubstituted aliphatic group, or a thiol protecting group. R^9 and R^{10} are each, independently, -H, a substituted or unsubstituted aryl group, a substituted or unsubstituted heteroaryl group, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aralkyl group, a substituted or unsubstituted heteroaralkyl group or an amine protecting group. Alternatively, R^9 and R^{10} taken together with the nitrogen to which they are attached form a heterocyclyl group. q is an integer from 1 to about 6. Each R^3 independently is a C_{1-6} alkyl group, preferably an isopropyl group. The phosphoramidite employed is commonly a betacyanoethyloxy-N,N-diisopropyl phosphoramidite.

Nucleoside bases include naturally occurring bases, such as adenine, guanine, cytosine, thymine, and uracil and modified bases such as 7-deazaguanine, 7-deaza-8-azaguanine, 5-propynylcytosine, 5-propynyluracil, 7-deazaadenine, 7-deaza-8-azaadenine, 7-deaza-6-oxopurine, 6-oxopurine, 3-deazaadenosine, 2-oxo-5-methylpyrimidine, 2-oxo-4-methylthio-5-methylpyrimidine, 2-thiocarbonyl-4-oxo-5-methylpyrimidine, 4-oxo-5-methylpyrimidine, 2-amino-purine, 5-fluorouracil, 2,6-diaminopurine, 8-aminopurine, 4-triazolo-5-methylthymine, 4-triazolo-5-methyluracil and hypoxanthine.

The nucleoside base may be protected. Examples of suitable protecting groups are well known in the art. Typically, nucleoside bases have amine groups which can be protected with an amine protecting group, such as an amide or a carbamate. For

example, the amine groups of adenine and cytosine are typically protected with benzoyl protecting groups, and the amine groups of guanine is typically protected with an isobutyryl group, a 4-isopropylphenoxyacetyl group or t-butylphenoxyacetyl group. However, other protection schemes, such as formamidine, may be used. For example, for fast deprotection, the primary amine groups of adenine and guanine are protected with phenoxyacetyl groups and the amine group of cytosine is protected with an isobutyryl group or an acetyl group.

It will be recognised that, whilst the formula (1) is expressed in terms of the natural, nucleosidic configuration (D-isomers), the present invention is equally applicable to the corresponding synthetic or unnatural configuration (L-isomers), to alpha and beta anomeric forms, and to mixtures of configurations.

The phosphoramidites which can be purified by the process according to the present invention are commonly the products of a reaction between a protected nucleoside comprising a free hydroxy group and a phosphitylation agent.

Phosphitylation agents commonly have the general chemical formula PG-O-PX^1X^2 wherein PG is as previously defined, and preferably a group of formula $-CH_2CH_2CN$; X^1 and X^2, which may be the same of different, represent leaving groups, such as halo, commonly bromo or chloro, or $-NR^{11}R^{12}$, wherein R^{11} and R^{12} each independently represents an alkyl, preferably a C_{1-6} alkyl, group, or R^{11} and R^{12} are joined, together with the N to which they are attached, to form a 5-7 membered ring. Commonly, at least one of X^1 and X^2 is a group of formula $-NR^{11}R^{12}$. Most preferably, X^1 and X^2 are the same, and it is particularly preferred that both X^1 and X^2 are $-N[CH(CH_3)_2]_2$ groups.

Examples of preferred phosphitylating agents include O-β-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite, (commonly known as "tetraphos"), O-β-cyanoethyl-N,N,N',N'-tetramethylphosphorodiamidite, O-β-cyanoethyl-N,N,N',N'-tetraethylphosphorodiamidite, bis (N,N-diisopropylamino)-2-methyltrifluoroacetylamino-ethoxyphosphine, bis (N,N-diisopropylamino)-2-diphenylmethylsilylethoxyphosphine and O-β-cyanoethyl-bis (N-morpholino) phosphorodiamidite.

The process according to the present invention is often carried out at a temperature in the range of from 0°C to about 50°C, and preferably at ambient temperature, such as from about 15°C to about 30°C.

The lower molecular weight impurities are predominantly comprised of decomposition and side reaction products of the phosphitylation agent. Commonly, the impurities have a molecular weight of less than about 375, and preferably less than about 350.

In certain embodiments, the solvent present in the phosphoramidite solution produced in the phosphitylation process can be subject to a solvent change in order to produce a solution which is compatible with a wider range of nanofiltration membranes. For example, where the phosphitylation process employs a chlorocarbon solvent,

especially dichloromethane, this can be exchanged for an alternative solvent, for example an ester, especially ethyl acetate. Further solvents which can be employed include ethers, such as tetrahydrofuran and dioxane, amides, such as dimethylformamide and N-methylpyrrolidinone, nitrile such as acetonitrile, and hydrocarbons such as hexane and toluene. A particularly preferred embodiment of the present invention comprises the nanofiltration of a solution of phosphoramidite in an ester solvent, especially ethyl acetate.

The phosphoramidite solution is advantageously treated, preferably prior to any solvent exchange, by contact with a basic solution, for example sodium carbonate solution, in order to neutralise acidic impurities.

Nanofiltration membranes that can be employed in the first aspect of the present invention are selected to be resistant to degradation by the phosphoramidite solution. Examples of nanofiltration membranes include those made from poly(ethylene), poly(propylene), poly(sulphones), poly(ethersulphones), poly(tetrafluoroethylene), poly(vinylidenedifluoride), poly(amides), poly(imides), poly(acrylonitriles), cellulose acetate and mixtures thereof. The membranes may comprise components immobilised onto a support, for example a silicone immobilised onto a poly(acrylonitrile) support. Particular examples are those membranes disclosed in US Patents 4,368,112, 4,748,288, 4,985,138, 4,990,725, 5,067,970, 5,093,002, 5,102,551, 5,205,934 and 5,265,734 and WO00/06293 (incorporated herein by reference). For the purification of nucleoside phosphoramidites, the membranes are commonly selected to have a molecular weight cut off at about 400. That is, the membrane allows the passage of compounds having a molecular weight of less than 400, but does not allow the passage of compounds having a greater molecular weight. Particularly suitable membranes are those disclosed in US5,264,166 (incorporated herein by reference).

In the process according to the present invention, "crude" solutions containing nucleoside or oligonucleotide phosphoramidite are pumped through the nanofiltration membrane, commonly using high pressure. The phosphoramidite is not permitted to pass through the nanofiltration membrane, whereas the lower molecular weight impurities are able to pass through. The nanofiltraton residues comprising the phosphoramidite can be washed with further fresh solvent. Commonly, the pumping across the membrane continues until the volume of phosphoramidite solution residue is significantly lower than the original "crude" solution, thereby simultaneously effecting purifying and concentrating the phosphoramidite. The process may be carried out using apparatus known in the art for nanofiltratio, and in particular using apparatus as disclosed in WO02/076588 (incorporated herein by reference).

The purified phosphoramidite may then be recovered from the residue by conventional methods.